ATTACHMENT 'B'

Short Technical Reports

Long-Range and Highly Sensitive DNase I Footprinting by an Automated Infrared DNA Sequencer

BioTechniques 23:300-303 (August 1997)

ABSTRACT

We have shown that an automated DNA sequencer is applicable to fluorescence-based detection of fragments in DNase I footprinting. We demonstrated the potential of long-range and highly sensitive DNase I footprinting taking advantage of an infrared-fluorescence automated DNA sequencer. Footprints of human transcription factor Sp1 were reproducibly detected ranging approximately between 100 and 750 bp on both strands of an 895-bp DNA fragment in a single electrophoresis run. We developed techniques in data collection and subsequent image processing for highly sensitive detection. Less than 0.1 footprinting unit (fpu; approximately 4.5 ng) of Sp1 was detected using 3.1 fmol of a 512-bp DNA fragment. This is greater than 10-fold increase in sensitivity over what has previously been reported by visible dye fluorescence DNA sequencers. This method will be very important in systematic analysis of transcription regulatory regions and in large-scale analysis of the transcription process.

INTRODUCTION

DNase I footprinting is frequently used to detect sequence-specific, DNA-binding proteins and to characterize their target sequences (2). Sample preparation by the solid-phase method has made DNase I footprinting faster and easier (7). Recently, an automated DNA sequencer was used to analyze fluorescein-labeled DNA fragments in a footprinting study (8). These new techniques suggest the possibility of automated analysis of multiple samples and of longer regions containing several sites of DNA-protein interaction in a single run. However, high background noise caused by autofluorescence of the gel and glass plates limits the sensitivity of detecting DNA fragments. High sensitivity is essential in long-range footprinting and in detecting rare transcription factors. Recently, fluorescent detection of DNA fragments labeled by an infrared dye (instead of a visible dye) showed an increase in the sensitivity by reducing the noise level (4,6,9).

In this work, as a model experiment, we investigate the potential of an automated infrared DNA sequencer in long-range and high sensitivity footprinting

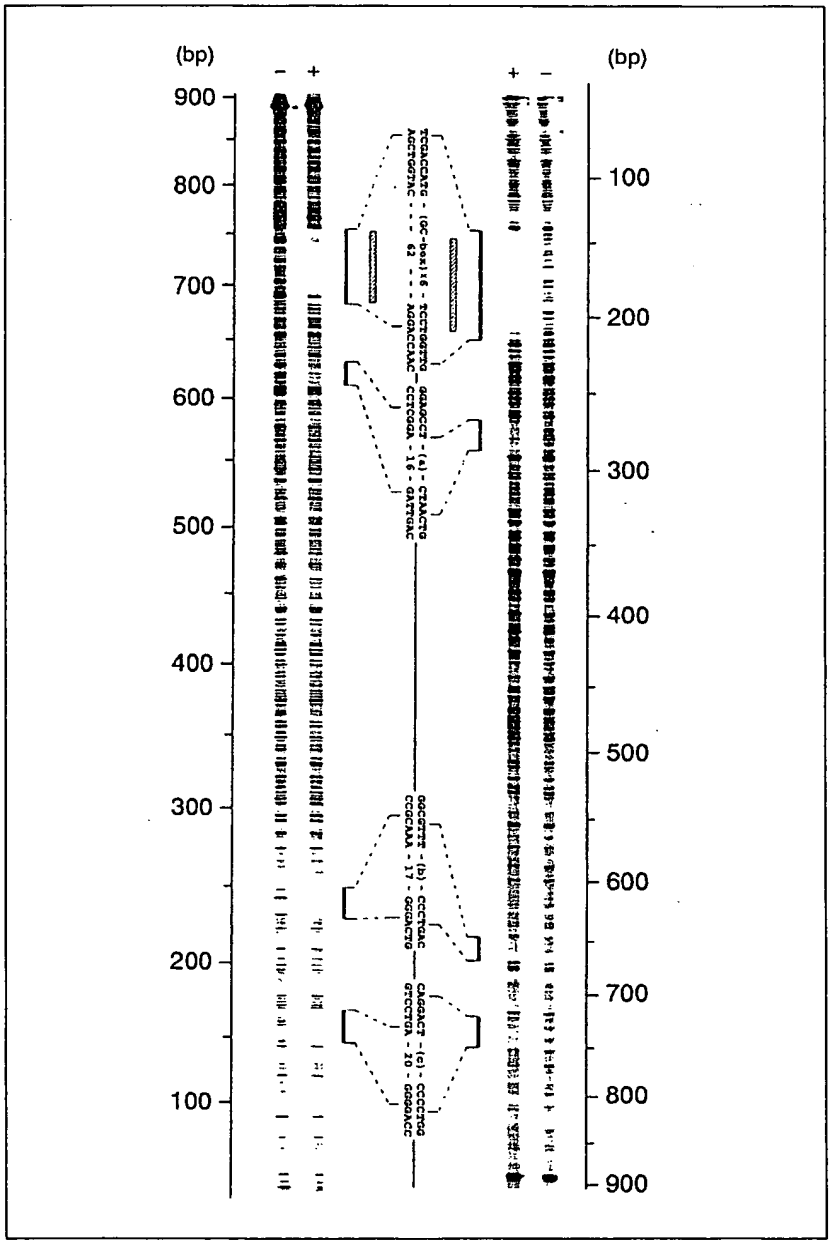

Figure 1. Long-range footprinting. One hundred fmol of a 895-bp DNA fragment amplified using primers, ps0599am (5′ CAGCTTGGAGCGAACGACCTACAC 3′) and sp6-lic (5′ GATTTAGGTGACACTATAG 3′) were used for footprinting. The fluorescent label was introduced by IRD41-labeled ps0599am and sp6-lic primer for the left and right panel, respectively. DNase I digestion was performed in the presence (+) or absence (-) of Sp1. Numbers at both sides of the footprints indicate nucleotide positions relative to the IRD41-labeled terminus, which was determined from the sequence ladder as a standard. Hatched boxes indicate the previously reported Sp1-binding region containing the six GGGCGG motifs (1). Solid black lines indicate regions where a footprint was detected in this experiment.

analysis using human transcription factor Sp1, which binds to the simian virus 40 (SV40) early promoter regulatory region (1). Footprints in the region of approximately 900 bp could be reproducibly scanned in a single run, and less than 5 ng Sp1 could be successfully detected by optimizing the data collection and the image processing conditions.

MATERIALS AND METHODS

Preparation of a Fluorescently Labeled DNA Fragment

DNA fragments containing Sp1 binding sites were prepared by polymerase chain reaction (PCR) using 50 ng of plasmid pSP72 containing the SV40 early promoter (Promega, Madison, WI, USA) as template. A combination of 5′-biotinylated and 5′-IRD41-labeled primers were used to introduce biotin and IRD41 fluorescent dye at different ends of the DNA fragments. The fragments were analyzed by electrophoresis on agarose gel before use and densitometrically quantitated after ethidium bromide staining with an ARGUS Image Analyzing System™ equipped with a UV-transilluminator (Hamamatsu Photonics, Tokyo, Japan). After purification by gel-filtration (MicroSpin™ S-400 HR; Pharmacia Biotech, Piscataway, NJ, USA), the biotinylated IRD41-labeled DNA fragments were immobilized on streptavidin-coated paramagnetic beads (Dynabeads® M-280-Streptavidin; Dynal, Oslo, Norway) according to the manufacturer's specifications. The fragment concentration was controlled so that 10 µg of magnetic beads contained 25 fmol of fragment, except that twice this amount of beads was used with less than 25 fmol of fragment to make handling easier.

Footprinting Reaction

Footprinting reactions were carried out according to the method described by Sandaltzopoulos and Becker (7) using 1 footprinting unit (fpu; approximately 50 ng) of purified recombinant Sp1 (Promega) per 35 fmol fragment. The buffer for the binding reaction consisted of 25 mM HEPES-NaOH (pH 7.8), 50 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 5% glycerol. After the completion of the reaction, the beads were suspended in 1.5 µL of loading buffer (95% formamide, 10 mM EDTA, pH 7.6, 0.1% xylene cyanol and 0.1% bromophenol blue). The sample was denatured for 2 min at 95°C and loaded on a sequencing gel (41-cm-length, 0.25-mm-thick, containing 5.5% Long Ranger Singel™ [FMC BioProducts, Rockland, ME, USA], 7 M urea and 0.6× TBE buffer [1× TBE buffer consists of 89 mM Tris, 89 mM boric acid and 2 mM EDTA], 0.5× TBE running buffer). It was electrophoresed for 3 h to analyze a 512-bp fragment (voltage, 2250 V; current, 30.6 mA; power, 68.8 W; heater temperature, 50°C; motor speed, 4; signal channel, 3; frames, 25) by a LI-COR® Model

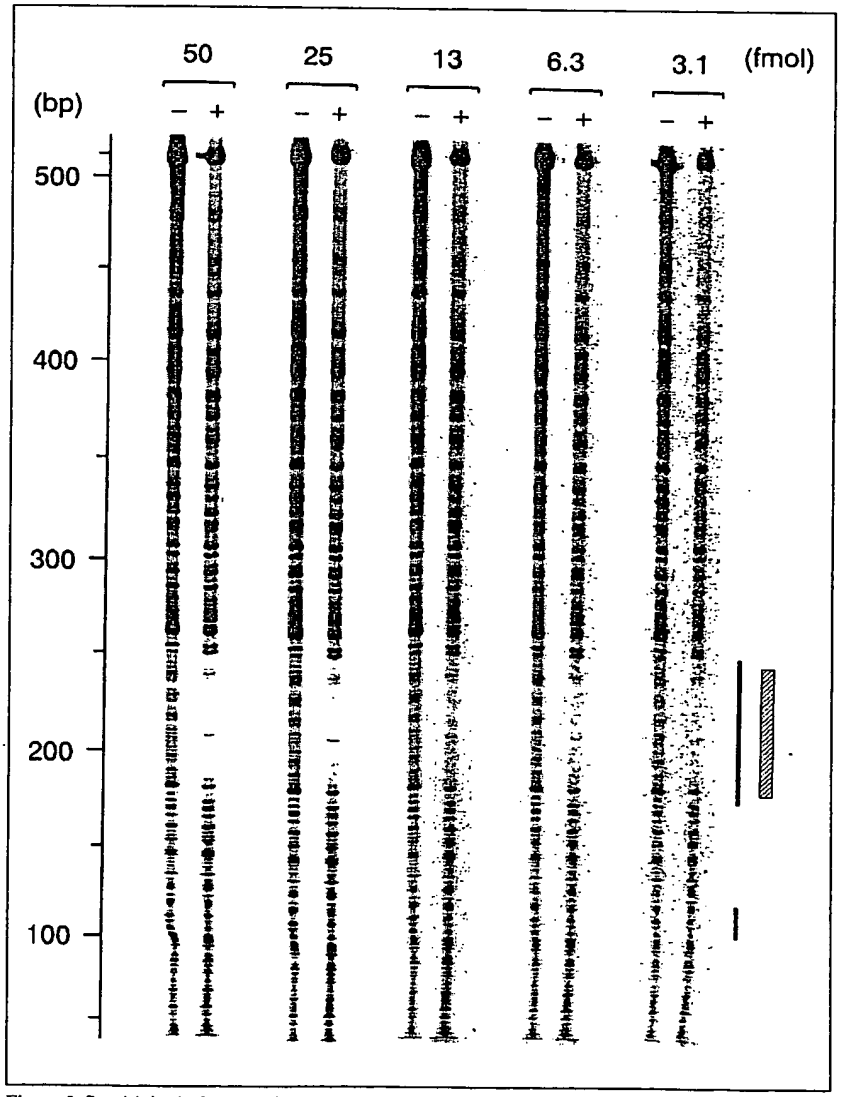

Figure 2. Sensitivity in footprinting by an infrared automated DNA sequencer. A 512-bp DNA fragment was amplified using primers, ps2323bp (5′ biotin-GTGTCGGGGCTGGCTTAACTATGC 3′) and T7 (5′ IRD41-GTAATACGACTCACTATAGGGC 3′). Indicated amounts of DNA fragment were used where the ratio of DNA fragment (fmol) and Sp1 (fpu) was fixed to 35:1. Signal enhancements were applied so that samples containing different amounts of DNA fragment could be seen with similar intensity. Because of minor over-digestion by DNase I in the presence of Sp1, the second weakly protected region (short solid black line) could not be detected until the ladder intensity around the region was re-equalized. Symbols are identical to those in Figure 1.

4000L DNA Sequencer™ with Base-ImagIR® Version 2.1 Software (Li-Cor, Lincoln, NE, USA). The 7-deaza-dGTP in the standard sequencing reaction (SequiTherm™ Long-Read™ Cycle Sequencing Kit-LC; Epicentre Technologies, Madison, WI, USA) was replaced with unmodified dGTP to prepare a sequence ladder as a molecular weight standard. The resulting 8-bit image was compressed 20% vertically by removing 4 lines out of 5, and the intensity was adjusted by Adobe® Photoshop® (Adobe Systems, San Jose, CA, USA).

RESULTS AND DISCUSSIONS

A strongly protected region derived from the Sp1-binding was clearly observed at the position reported previously (1) on both strands of the 895-bp DNA fragment as shown in Figure 1 (hatched box). Four clear gapped regions were observed, including the strongly protected and three other weakly protected regions, ranging approximately between 100 and 750 bp from the fluorescent-labeled terminus (solid black bars). Each gapped region on one strand was also observed on the complementary strand at the same position. A very weakly protected region appeared to exist at the region 691–714 nucleotide (nt) in the right strand and the corresponding region 185–206 nt in the complementary strand. This example clearly indicates that our method is powerful enough to analyze protein binding to a 1000-bp fragment in a single run with high reliability.

We examined the sensitivity of the infrared DNA sequencer in footprinting by using the 16-bit data collection mode of the LI-COR Model 4000L DNA Sequencer. When 16-bit data were collected, signal gain and offset were manually adjusted to 25 and 93, respectively. These values were determined by pre-scanning as was done by McIndoe et al. (3). The linear conversion from 16-bit to 8-bit data in tag image file format (TIFF) was made by software developed for this work to visualize the images on a video display in 256 gray scales and to facilitate processing of the image by commercial software such as Adobe Photoshop. During the conversion process, the optimized gain and offset were applied to the image data using appropriate look-up tables. Generally, pixels ranging in value between 3072 and 4096 digits in 65 536 of the 16-bit data width were converted into an 8-bit image using 4 digits in a 16-bit image as one digit in the 8-bit image, resulting in 256 gray scales. The image quality could not be further improved by modifying the look-up table due to background fluorescence fluctuation (approximately 58 digits width).

As shown in Figure 2, the footprints of the strongly protected region (long solid black line) could be clearly observed in quantities of DNA fragment ranging from 50 to 3.1 fmol, detecting from 1.4 to 0.089 fpu (approximately 70 to 4.5 ng) of Sp1. Although the image quality was reduced according to the decrease in the amount of DNA fragment, the signal-to-noise ratio was still adequate to find the gapped region in the experiment with 3.1 fmol of DNA fragment. Our method shows a greater than 10-fold increase in sensitivity with a fragment nearly twice as long as that reported previously using a visible fluorescence DNA sequencer (5). The signal-to-noise ratio of the 16-bit footprinting data was approximately 4 times higher than the data collected in 8-bit mode (data not shown). Since the minimum signal-to-noise ratio of the results of the 895-bp fragment (Figure 1) was approximately one-third that of the 8-bit data with the same amount of 512-bp DNA fragment used (Figure 2), the detection limit of the 895-bp DNA fragment is calculated to be approximately 10 fmol when collected in 16-bit mode. We have found that a smoothing algorithm that replaces the intensity of a pixel with the average intensity of a pixel and surrounding pixels, could increase the signal-to-noise ratio by approximately twofold without a significant reduction of image quality. Integration of five horizontal lines instead of picking one line out of every five lines during the vertical image compression may further improve the sensitivity.

Although we used 16-bit data collection to obtain the highest sensitivity, a wide dynamic range was not thought to be essential, since 256 gray scales are adequate to visualize a high-quality image. However, during these experiments, we realized that a wide dynamic range is important in footprint detection. The band intensity may vary significantly depending on position in the footprint ladder, especially when a long DNA fragment is used. Sixteen-bit data collection mode gives the most reliable footprinting results both in regions of weak and strong signals. The process of converting the data using look-up tables is very similar to varying the exposure time in a radioisotope experiment.

Footprinting by an automated infrared DNA sequencer was demonstrated to have potential for both 1000 bp long-range analysis and for sensitive detection comparable to using radioisotopes. The entire footprinting experiment can be done in one day, including DNA fragment preparation by PCR and image processing. Our method will be very important in a systematic analysis of the transcription regulatory region of a large number of genes, enabling a large-scale analysis of transcription process.

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We would like to thank Mr. Lyle R. Middendorf and Mr. Charlie Troxel of Li-Cor for helpful discussions and comments on the manuscript. Address correspondence to Masayuki Machida, Department of Molecular Biology, National Institute of Bioscience and Human-Technology, Higashi 1-1, Tsukuba, Ibaraki 305, Japan. Internet: machida@nibh.go.jp

Received 27 September 1996; accepted 2 February 1997.

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